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Cell cycle dependent toxicity of an amphiphilic synthetic peptide

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Abstract The cytotoxic properties of an amphiphilic synthetic peptide are presented. Comparative analysis of proliferating, differentiated and confluent H9C2 adherent cells and L1210 cells in suspension shows a correlation between toxicity and cell stage (proliferating cells). Electrophysiological measurements on *Xenopus laevis* oocytes bathed in the peptide also demonstrated the induction of cationic currents, which is voltage and phosphate dependent. These results allow us to hypothesize that the observed toxicity is related to membrane hyperpolarization of proliferating cells at the G1/S cell cycle phase transition.

Key words: Peptide toxicity; Cell cycle; Differentiation; Cationic current

1. Introduction

Synthetic peptides can be used to vectorize therapeutic molecules [1–3]. Their role involves crossing the plasma membrane (and sometimes the nuclear membrane) to carry an active molecule into the cell. Often such peptide vectors present strong toxicity in cells [4,5].

The reason for peptide toxicity is most often intrinsically related to the design of the peptide, or a part may be soluble in the phospholipid membrane as a transient anchor. More subtle additions to the design may include a hydrophilic region which improves aqueous solubility and the half-life of membrane residence. The resulting peptide might stay sufficiently long in the membrane, before crossing into the cytoplasm. Two properties may thus originate from the amphiphilic construction: (i) in aqueous environments the peptide has a great propensity to self-aggregate [6], (ii) in phospholipid environments, the structure of the membrane bilayer may be disturbed (by the peptide itself, its flip-flop action, membrane aggregation and/or destruction of the bilayer itself). Without taking into account more biological properties of the peptide (for example, interference in phosphorylation cascades), the aforementioned properties result in toxic behavior; generally, the peptide is discarded as too dangerous for therapeutic use. At present, drugs (taxol, anthracyclins, etc.) are more generally used for killing proliferating cells. Amphiphilic peptides could be interesting tools, especially if they target proliferating cells [4,7]. The aim of this paper is to present a peptide designed to be used as a vector, but showing marked toxicity which preferentially targets proliferating cells.

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2. Material and methods

2.1. Dioleylphosphatidylglycerol vesicles

Large unilamellar vesicles (LUV) were prepared from commercial dioleylphosphatidylglycerol (Sigma). Original solvent ($CHCl_3$) was evaporated under a stream of nitrogen and desiccated under high vacuum for at least 3 h to remove the residual solvent, then DOPG was solubilized in chosen buffer. The resulting solution was sonicated (~3 min 3 times at 80% pulse cycle in an ice bath) with a probe sonicator.

2.2. Circular dichroism

Dichroism spectra were recorded on a Mark V (JOBIN YVON, Paris) dichrograph equipped with a thermostatically controlled cell holder, and all spectra were recorded at 25°C. The cell (1 mm thin) used had been extensively washed with sulfochromic mixture and rinsed with double-distilled water to avoid the adsorption of the peptide. The spectra shown are the mean values of five spectra. The scan rate was 20 nm/min and the data point number by scan around 650. Smoothing, structure assignation and percentage come from Greenfield et al. [9] and Dicroprot 2.3d software (G. Deleage, Lyon).

2.3. Fluorescence intensity

The emission spectra were obtained on a Spex 2000 (JOBIN YVON, Paris), the apertures being 2 mm for both slots, excitation and emission, and a high voltage of 950 V was applied. In all cases, the baseline corresponding to the buffer and DOPG vesicles was subtracted from the given spectra.

2.4. Cell culture and toxicity evaluation

Rat embryo myoblasts (H9C2) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% or 1% (in the case of differentiation assays) fetal calf serum (FCS). The cells (volume of medium 100 μl) were plated on glass coverslips and incubated in 35 mm cell culture boxes (Nunc) for 24–48 h at 37°C. The peptide was added in 100 μl phosphate buffer 50 mM, pH 7.3. Toxicity was evaluated by determining trypan blue (Sigma Chemicals) exclusion versus a non-treated control. Murine lymphocytes (L1210) were cultured in RPMI (+Gln) medium supplemented with 8% FCS. Cells were suspended in 1.5 ml medium in 35 mm cell culture boxes. FACS measurements were performed after DNA staining using solution of PBS-propidium iodide (0.1 mg/ml)-Triton X-100 (0.3%). The filters used (FL2A) were 530/617 (excitation/emission, respectively).

2.5. Electrophysiological recording on Xenopus laevis oocyte

Ovaries were surgically removed from *Xenopus laevis* females (Elevage de Lavalette, Montpellier, France) and anesthetized using a 0.2% MS222 solution (Sigma Chemicals). Oocytes were used after mechanical dissociation and extensive washing in OR-2 solution (in mM: NaCl 100; $MgCl_2$ 2; KCl 2; $CaCl_2$ 1.8; HEPES 10; pyruvic acid 2.5; gentamicin 50 μg/ml, neutralized at pH 7.2 using NaOH). Whole cell currents were recorded under two electrode voltage-clamp using the GeneClamp 500 amplifier (Axon Instr., Burlingame, CA). Current and voltage electrodes were filled with: CsCl 2.8 M; BAPTA 10 mM, pH 7.2; with CsOH. The batch-clamp headstage was connected to the bath using two agar bridges filled with 2% agar in 3 M KCl. Before each recording, the electrode resistance was checked, and if necessary adjusted to less than 1 MΩ, and the liquid junction potential was nulled. The typical recording solution (Na100) had the following composition (in mM): NaCl 100; HEPES 10; $MgCl_2$ 2; pH 7.2 with NaOH. Ionic selectivity was tested using several solutions. Currents were filtered and digitized using a DMA-Tecmar labmaster and subsequently stored on a IPC 486 personal computer using the version 6.02 of the pClamp software (Axon Instr.). Voltage

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ramps (from −60 mV to +50 mV at 275 mV/s) were applied from a holding potential of −40 mV, −60 mV, −80 mV or −100 mV every 5 s. The peptide was dissolved in an appropriate solution at 10 μM and added directly to the recording chamber (250 μl). Junction potentials between the different solutions were less than 1 mV.

2.6. Chemicals

The peptide was synthesized using Fmoc strategy as described previously [8]. The sequence of the peptide used was: NAc-GALFLGWLGAAGSTMGAWSQPKKKRKV-Cya, where NAc is the acetylated N-terminus and Cya is the cysteamide group on the C-terminus. DOPG, solvents and buffer contents were purchased from Sigma Chemicals (St Quentin Fallavier, France).

3. Results

3.1. Secondary structure

Fluorescence and circular dichroism studies were used to determine the peptide structure in a lipid environment. DOPG vesicles were chosen because they greatly reduce the light diffusion phenomena due to the high concentrations of lipid needed to reach the ratio Ri = 20 or Ri = 40 (see below), since they are transparent, in spite of their minor contribution to animal cell membranes. In the presence of liposomes, the peptide is incorporated into the phospholipid bilayer as shown by the fluorescence intensity measurements. Once excited at 285 nm, the tryptophyl residues have an emission with a maximum wavelength shift from 353 to 338 nm (Fig. 1B) which indicates a less polar environment for the chromophore [10]; the peptide can be considered to be embedded in the lipid bilayer. The peptide contains two tryptophans, one in the hydrophobic region (W7), the other near the hydrophilic region (W18), so the shift of the maximum wavelength is considered to be a mean value reflecting the two possible situations with respect to the bilayer.

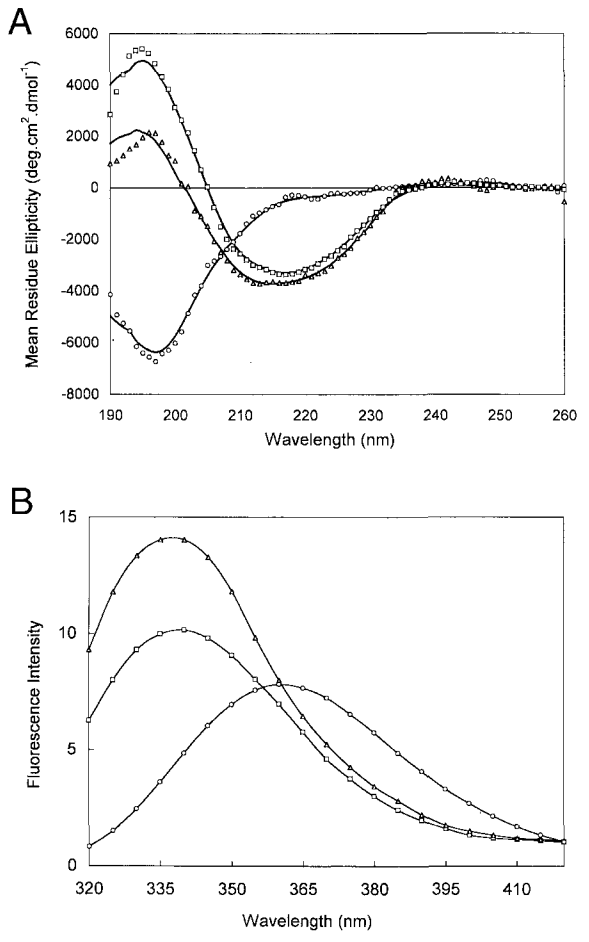

Fig. 1. A: Circular dichroism spectra (symbols = experimental, plain line = simulated) of 2×10^{-5} M peptide in aqueous medium: KH_2PO_4 20 mM; pH = 7.2) ○, and in presence of LUV (DOPG) in KH_2PO_4 20 mM, pH = 7.2. The ratio Ri = [lipid/peptide] is 20 (□) or 40 (△). B: Peptide fluorescence spectra corresponding to each solution shown in A, without DOPG (○), for Ri = 20 (□) and Ri = 40 (△).

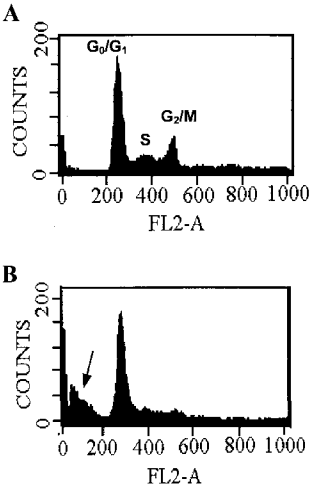

Fig. 2. FACS analysis of L1210 cells. A: Standard propidium iodide staining assay showing three phases of cell cycle. B: Analysis of the peptide effect (10 μM) after 24 h incubation. There is a persistence of the G0/G1 peak and a dramatic decrease of the two other populations. Much cell debris appears (arrow).

Under the same conditions, CD results show that the peptide adopts mainly a β conformation in the presence of liposomes, as indicated by the minimum occurring around 215 nm (Fig. 1A). In aqueous solution, mainly a random coil conformation is observed with a typical spectrum (Fig. 1A) showing a minimum at 197 nm. The software used allows the determination of four major components: helix (h), sheet (s), turn (t) and coil (c); explicit results come from the Fasman algorithm: Without vesicles (○): h = 7.4%, s = 14.8%, t = 0%, c = 77.8%; Ri (lipid/peptide) = 20 (□): h = 9.5%, s = 76.2%, t = 0%, c = 14,3%; Ri = 40 (△): h = 10.9%, s = 61%, t = 0%, c = 28.1%.

3.2. Toxicity

3.2.1. L1210. Preliminary experiments indicated that the peptide exerts toxic effect on murine L1210 cells. Fig. 2 shows a FACS analysis in which a substantial part of the cell population is killed. A decrease of G2 and S peaks (the latter being more affected) can be observed.

3.2.2. H9C2. On the basis of these results, a more convenient cell line was chosen which can be more easily studied (adherent), not only under proliferative conditions, but also in confluent and differentiated states.

3.2.3. Proliferating H9C2. The cell cultures were incubated with the peptide solution when they reached 70% confluence. Two experimental conditions were studied: 1% or 10% FCS, which correspond to two different proliferation rates. As shown in Fig. 3, the toxicity is more marked for

the more rapidly proliferating cells (10% FCS; Fig. 3B) than for less rapidly proliferating cells (1% FCS; Fig. 3A). It depends on at least two other factors: (i) the peptide concentration and (ii) the contact time (1–3 days). After 3 days with a 10 μM initial peptide concentration, 100% of the cells were killed in 1% or 10% FCS.

3.2.4. Differentiated H9C2. Serum deprivation induces H9C2 cell differentiation into myotubes [11]. Once the cells were differentiated (morphological phenotype), the peptide was added to the external medium, as a test to relate its toxicity after exit from the cell cycle. Under these conditions, the rate of cell death in cells induced to differentiate is about 10%, roughly equivalent to the rate of cell death in untreated control cells. Thus as the cells leave the cycle and differentiate, no peptide induced toxicity is observed.

When the H9C2 cells reach 100% confluence (a contiguous and continuous layer on the coverslip), the rate of peptide induced toxicity falls to about 30% of that observed in proliferating (10% FCS) H9C2 cells (data not shown). This interesting point is discussed further.

3.3. Electrophysiological tests

One possible explanation for this peptide induced toxicity could be cell membrane depolarization by the creation of pores. To test this hypothesis several electrophysiological measurements (whole cell current) were undertaken on *Xenopus laevis* oocytes. The application of peptide to voltage clamped oocytes induced strong currents showing a potential reversal of ~−7 mV in typical 100 mM NaCl working solutions (Fig. 4A). Replacing Na^+ and Cl^- with different cations and anions, respectively, induced a potential reversal series of currents, suggesting that the channels formed by the peptide were mainly for cations, without marked selectivity (data not shown). It is interesting to note that changes in membrane conductivity induced by the peptide were strongly voltage dependent, being larger for hyperpolarizing potentials (data not shown). This voltage dependence was lower when using phosphate ions in the external solution. Once induced, the membrane current did not reach a steady-state value and often oversaturated the capacity of our amplifier. Washing out the peptide did not reverse the effect, but led to a stabilization of the peptide induced membrane current (Fig. 4B). Reversibility was only obtained when divalent cations (Ca^{2+} or Ba^{2+} 2 mM) were added to the bathing solution, suggesting an action of these ions on channel formation or gating stability. The gating potential rises from −90 mV to −60 mV as the phosphate ions are present in the external medium. Furthermore, calcium ions reverse the conductance to zero; the normal behavior is then recovered by washing the oocyte with the working solution (2 mM $CaCl_2$ added) (Fig. 4B).

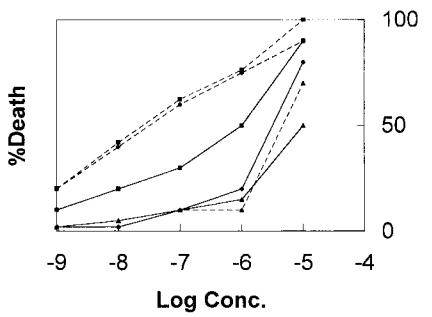

Fig. 3. Toxicity evaluation on H9C2 cells (as percent death). Treatment was for 24 h (▲), 48 h (♦) or 72 h (■). A: With DMEM, supplemented 1% FCS. IC_{50} remains between 1 and 10 μM. B: With DMEM, supplemented 10% FCS. IC_{50} is unchanged for 24 h. At 48 h and 72 h toxicity is dramatically increased (IC_{50} close to 10 nM).

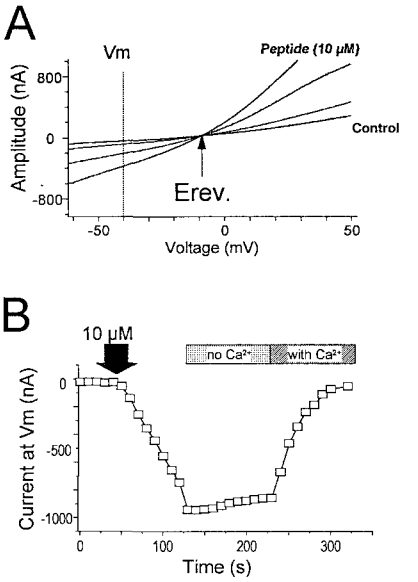

Fig. 4. A: Starting from a steady potential of −60 mV, a potential ramp is applied every 5 s. E_{rev} is the current reversal potential characteristic of the channel selectivity (see text). B: Current was measured at −40 mV (Vm, part A) during a series of voltage ramps similar ro part A. Addition of 10 μm peptide induced a marked increase of current at this potential, which did not reach a steady state with time. Wash out of the peptide (no Ca^{2+} solution) stabilized this increase without reversibility. Reversibility could only be obtained if divalent cations were added to the washing solution (with Ca^{2+} solution).

4. Discussion

The problem of examining the effects of peptides in mammalian cells in culture is the requirement that the cells be physically broken, trypsinized or fixed. Consequently, global observations on quiescent cells such as *Xenopus laevis* oocytes seem to be more pertinent and informative. The electrophysiological measurements were undertaken to look for a possible role of the peptide in relation to membrane properties. It is known that membrane proteins such as porins (for example phosphate specific) are responsible for pore formation [12–17]. Such proteins are constructed as 16 antiparallel β strands [14]. When embedded in phospholipid membranes, the induced β strand formation of the peptide under study may be analogous to the porins due to aggregation of the peptide molecules probably caused by an electroneutralization of positive charges, followed by the chain-chain interaction of the hydrophobic part (N-terminal). Such a structure may be possible within the structure of the membrane.

In *Xenopus laevis* oocytes, we observed the induction of non-specific cationic currents. The addition of phosphate buffer in the medium raised the gating potential from −90 to −60 mV. This latter value is a more normal level for mem-

brane potentials than −90 mV. Furthermore, currents are reversed to zero by addition of calcium ions. The inhibition of the monovalent cation current by Ca^{2+} could be explained, for example, by a blocking behavior with respect to the hydrated radius of cations. No calcium current was detected, thus supporting the idea of a channel with a well defined pore dimension or a competition between Ca^{2+} and basic residues with respect to the phosphate ions of the lipids. Mg^{2+}, which is much more hydrated than Ca^{2+}, does not show any effect on current formation.

In summary, (i) toxicity is observed on proliferating cells as a β structure exists in bilayers, (ii) this toxicity targets proliferating cells and to a lesser extent confluent cells, but not quiescent or differentiated cells, (iii) the G1/S transition is probably an important event, (iv), cationic voltage gated currents are observed in presence of the peptide.

It is noteworthy that a relationship exists between the cell cycle and plasma membrane polarization. The relationship between K^+ channels [18] and cell proliferation has been described in several cell lines including T lymphocytes [19], sea urchin embryos [20], human mammary tumor cells (MCF-7) [21], Chinese hamster lung cell (V79) [22], human melanoma cells (IGR-1) [18], tumor cells from the anterior pituitary gland (GH3) [23] and neuroblastoma cells (Neuro-2A) [24]. The membrane potential strongly depends on the K^+ channel state [21,22]. Furthermore, during the cell cycle, both K^+ channels and membrane potential vary; a membrane hyperpolarization occurs in proliferating cell lines, particularly at the G1/S phase transition [19,21,23], due to the opening of type n K^+ channels and their increased number [19]. The current opinion is that calcium ions are released (1–10 μM) [19] from internal stocks, which initiates the calcium dependent cell cycle events including phosphorylation of $p34^{cdc2}$ protein. [18,20]. Membrane potential rises at the G1/S phase boundary and it remains high throughout the G2 and M phases [22,23].

As a relation exists between outward K^+ current and internal Ca^{2+} release, a simple hypothesis could be that cationic input current due to the peptide cancels the K^+ output current due to the G1/S transition. Internal Ca^{2+} is released, possibly until total depletion. Concurrently, calcium dependent phosphorylation of cyclins may be elevated. This mechanism may be true if the membrane potential rises to more negative values, i.e. when the cells synthesize DNA. The membrane potential is not always negative enough to reach the gating potential; the phosphate ions change this gating potential toward less negative values (by 30 mV in *Xenopus laevis* oocytes), so that most proliferating cells fall into this category and die. On the other hand, the observation on contiguous mitotic and non-mitotic cells shows that their membrane potentials are coupled; the membrane potentials of mitotic contiguous cells have less negative values than those of isolated mitotic cells [22]. A similar observation has been made with respect to the maturation of macrophages between blood and body tissue [19]. The preceding argument explains the decrease in toxicity (30%) observed in confluent cells, with respect to proliferating non-confluent cells.

The resulting peptide toxicity was also observed in synchronized cells (REF 52) in the course of vectorization experiments. No cell death was observed when contact occurred in the G0/G1 and S phases, while an almost complete destruction of the cell cultures occurred when the peptide was applied in the late S, G2 or mitotic phases (results not shown). This result reinforces the relation between toxicity, proliferation and cell cycle in another cell line.

5. Conclusion

The toxicity of the synthetic peptide studied seems strongly related to the cell cycle, more precisely to the hyperpolarization occurring at the G1/S transition. The explanation given as a first hypothesis concerns the inward cationic current induced by the peptide which counterbalances K^+ efflux current and the subsequent internal calcium mobilization. This hypothesis has to be strengthened in future studies and extended to more cell lines. Nevertheless, it is clear that the toxicity targets proliferating cells.

One might hope that this kind of toxicity could be used in vivo to kill certain cancerous cells, particularly metastatic cells. We propose to analyze this possibility in future work by studying the same cancerous cell line in vitro and in vivo. The fine understanding of the mechanism by which the toxicity acts may guide us toward a design of a molecule mimicking the peptide used in this work.

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